

The University of Chicago

RELATION OF HEMOTOXIN PRODUCTION TO METABOLIC ACTIVITIES OF STAPHYLOCOCCI

A PART OF A DISSERTATION SUBMITTED
TO THE FACULTY OF THE DIVISION OF
THE BIOLOGICAL SCIENCES IN CANDIDACY
FOR THE DEGREE OF DOCTOR OF PHILOSOPHY

DEPARTMENT OF HYGIENE AND BACTERIOLOGY

1933

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JOSEPHINE MCBROOM

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RELATION OF HEMOTOXIN PRODUCTION TO METABOLIC ACTIVITIES OF STAPHYLOCOCCI

JOSEPHINE McBROOM

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The question is unsettled as to whether certain so-called toxic properties of microorganisms, among which is their ability to produce hemotoxin, are associated with any demonstrable fundamental physical and chemical activities of the organisms. The following experimental survey of certain metabolic activities of staphylococci was undertaken with the purpose of determining whether there exists any association between these activities and the hemotoxic properties of the organisms.

Activities representative of a number of fundamental types of metabolism have been studied, namely, oxidation-reduction (reducing power), proteolytic, lipolytic and carbohydrate utilizing properties. Tests used in the measurement of the various activities were:

1. Oxidation-reduction, reduction of methylene blue to its leuco-base
2. Proteolytic activity, hydrolysis of peptone with determination by formal titration of amino acids liberated
3. Lipolytic activity, hydrolysis of tributyrin with determination of amount of acid formed, and
4. Carbohydrate metabolism, fermentation of glucose with measurement of acid formed.

Staphylococci were used in the study because they produce easily measurable, stable, hemotoxic substances which are closely associated in titer with strength of other toxic properties (i.e., dermatotoxin and "killing toxin" for rabbits¹ and because their hemotoxic properties may be readily and markedly enhanced by cultivation in an atmosphere containing carbon dioxide.²

METHODS

Bacterial preparations.—Whole cultures of staphylococci were used in the studies recorded in tables 1 and 2. Preliminary attempts were made to demonstrate enzymic activities (proteolytic, lipolytic and glucose fermentation) of Berkefeld N filtrates. Filtrates of broth cultures of the 4 strains of table 1, frozen and thawed repeatedly ten times before filtration, exhibited activity similar to but weaker than that demonstrable in whole cultures, and too slight to be considered beyond the scope of experimental error. Whole culture preparations used accordingly in subsequent experiments were prepared in two ways, (a) broth cultures, used in measurement of reducing power for methylene blue, (b) soft agar extracts, used for measurement of

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1. Burnet, F. M.: J. Path. & Bact. **32**: 717, 1929; Weld, J. T. and Gunther, Anne: J. Exper. Med. **54**: 315, 1931.

2. Burnet, F. M.: J. Path. & Bact. **33**: 1, 1930.

proteolytic and lipolytic activities and glucose-decomposition. Whole broth cultures consisted of 24 hour cultures in veal infusion broth (pH 6.8), 10 cc. quantities in ordinary test tubes, inoculated with 0.5 cc. of a 24 hour broth culture of the organism. Soft agar extracts were prepared by inoculating a semisolid medium consisting of 1% veal infusion agar (pH 6.0) in 75 cc. amounts in Kolle flasks with 1 cc. of a 24 hour broth culture of the organism. After incubation for 48 hours, the liquid was pressed out with sterile precautions and the fluid containing the bacterial culture was used immediately for testing.

Hemolytic tests.—To the bacterial preparations in 0.5 cc. volume (undiluted, and diluted to give final concentrations of 1-40, 1-100, 1-200, 1-400, etc.) were added 0.5 cc. of a 2% suspension of washed rabbit corpuscles. Tests were read after 2 hours incubation at 37 C. and after over-night refrigeration. The unit of hemotoxin was taken as the highest dilution of hemolysin which caused complete hemolysis of the resulting one per cent suspension of rabbit corpuscles. For tests of undiluted preparation the extent of hemolysis is recorded as (0, +, ++, +++, +++++).

Protease activity—hydrolysis of peptone.—The extent of hydrolysis of peptone to amino acids by the enzymic preparation (whole soft agar extracts) was determined. Brown's modification of the Sørensen method of formol titration was used.³ Hydrolysis produced in 4% peptone solution after 60 hours exposure to whole broth cultures was determined. The amount of NaOH necessary to neutralize the amino acids liberated was measured by testing the enzyme substrate mixture before and after incubation. The difference between the initial and final readings represents the amount of peptone hydrolyzed to amino acid by the enzymic preparation.

The enzymic substance to be tested was added in quantities of 1 cc. to 10 cc. amounts of a 4% solution of Witte's peptone (pH 7.0). Immediately after mixing, 1 cc. of the enzyme-substrate mixture was removed, transferred to a 1×8 inch test tube, and the volume made up to 10 cc. with neutral distilled water. Five drops of 0.02% phenol red indicator were added and the solution brought to a pH of 8.0 with N/40 NaOH. To it were added 8 cc. of neutral formol and 4 drops more of 0.02% phenol red. The tube was whirled gently, care being taken not to shake thereby absorbing carbon dioxide from the air. Enough N/40 NaOH (the exact quantity being noted) was immediately added to readjust the mixture to pH 8.0.

The original enzyme substrate mixture was incubated for 60 hours. At the end of that time 1 cc. was again tested as above. The differences between the amounts of NaOH required in the initial and the final tests represent the amount of NaOH necessary for neutralization of the amino acid groups in the enzyme substrate mixture. Control experiments with the enzymic substance in neutral distilled water in place of the peptone substrate represent the amount of NaOH required to neutralize acid formed due to protein decomposition, etc., in the enzymic substance itself. This quantity of NaOH was subtracted from the amount obtained in the enzyme substrate mixture to represent the final quantity required to neutralize the acid formed

3. J. Bact. 8: 245, 1923.

due to enzymic activity alone. Results are expressed in milligrams of nitrogen freed per 100 cc. of medium.

Lipase activity—hydrolysis of tributyrin.—The extent of hydrolysis of tributyrin to butyric acid and glycerol was determined by measurement of amounts of NaOH necessary to neutralize acid formed after 60 hours exposure of 2% tributyrin to the enzymic substance (whole bacterial culture). The culture to be tested was added in quantities of 1 cc. to a 2% emulsion of tributyrin (pH 7.0) in 10 cc. amounts. Immediately after vigorous mixing 1 cc. of the enzyme substrate mixture was removed, transferred to a test tube and the volume made up to 10 cc. with neutral distilled water. Five drops of 0.02% phenol red indicator were added, burette readings taken, and the solution brought to a pH of 8.0 with N/40 NaOH, noting the exact quantity of NaOH required. This procedure was repeated after incubation of the enzyme substrate mixture for 60 hours. The differences between the amounts of NaOH required before and after incubation represent the cc. of NaOH* required to neutralize the acid formed in the mixture. Controls with the enzymic solution in neutral distilled water in place of the tributyrin substrate represent the amount of NaOH required to neutralize acid formed due to protein decomposition, etc., in the enzyme substance itself. This quantity of NaOH was subtracted from the amount obtained in the enzyme substrate mixture in order to represent the final quantity of NaOH necessary to neutralize the acid formed due to enzymic activity alone.

Glucose decomposition.—The extent of utilization of glucose with formation of various acids was determined by measurement of quantities of NaOH necessary to neutralize acid formed after 60 hours of exposure of 4% glucose to the enzymic substance. The procedure for testing was exactly similar to that used in the determination of the extent of hydrolysis of tributyrin, the only difference consisting in the substrate used (glucose in place of tributyrin).

Duplicate tests were made with each enzyme substrate and control mixture for checking of tests and burette readings. Only those were used in which the duplicate reading differed from the original by not more than 0.04 cc. The average of the two readings was taken as the final result.

Reduction of methylene blue.—A stock solution of medicinal methylene blue was prepared in dilution of 1–1,000 in distilled water, autoclaved, and stored in the icebox until time for use. Immediately before performing the test, portions of the solution were diluted to the desired concentration. Five-tenths cc. of the diluted solution was mixed with 0.5 cc. of the bacterial preparation in 8 to 9 mm. tubes, making a volume of 1 cc., and final dilutions of 1–2,000 and 1–10,000. The tubes were sealed immediately with vaseline, and examined for loss of color with the production of the leuco-base of methylene blue after incubation at 37 C. for 30 minutes, 1, 2, 4, and 24 hours. The strength of reductase activity is indicated by the extent of reduction occurring in the tubes (0, +, ++, +++, +++++), and by the speed with which it occurred.

Results recorded in the body of this paper show that, by the use of methylene blue in testing reducing power, a distinction appears between hemotoxic

and nonhemotoxic strains. It is to be stressed that the distinction is a relative, not an absolute one. With very dilute methylene blue (1–10,000) all the strains, hemotoxic and nonhemotoxic, caused some visible reduction, slower in many instances in nonhemotoxic strains but not affording the distinct differentiation of more concentrated solution. In the interest of brevity only results at the optimum differentiating concentration of methylene blue (1–2,000) are reported here. Furthermore, entire uniformity in technic, with as little manipulation of cultures as possible, must be observed in order to obtain comparable results. Enhancement of hemotoxin production by use of soft agar extracts cultivated in an atmosphere containing CO₂ was therefore found impracticable.

All the broth preparations tested exhibited heavy growths of the organisms. Bacterial counts performed upon a few of the preparations showed no marked difference in numbers of organisms. Fairly uniform turbidity occurred in most cultures. Certain of the nonhemolytic strains appeared, however, to yield slightly less turbid but still abundant growths. Differences in numbers of bacteria produced has therefore not been entirely eliminated as a factor in the differences of extent of reducing power. The pronounced distinction between hemotoxic and nonhemotoxic strains, together with the abundant growths obtained in all cultures, render it unlikely that the number of organisms produced is of significance in the differentiation.

PROTEOLYTIC, LIPOLYTIC AND GLUCOSE-UTILIZING ACTIVITIES

Under the experimental conditions described, results in table 1 demonstrate no pronounced or consistent differences in extent of peptone hydrolysis, tributyrin hydrolysis, or glucose fermentation, (a) between hemotoxic and nonhemotoxic strains of staphylococci, and (b) between very strongly hemotoxic preparations (400 to 800 units) obtained by cultivation in 20% carbon dioxide and air, and normally hemotoxic preparations (2 units) prepared by ordinary aerobic cultivation. Stronger enzymic activities occurring in certain of the hemotoxic preparations appear to bear no specific relation to hemotoxic activity for the reasons that, (a) the differences are of no great magnitude, (b) differences of similar magnitude appear in certain preparations exhibiting no increased hemotoxin upon carbon dioxide cultivation as well as in those exhibiting markedly increased hemotoxin. Similar lack of correlation of hemotoxin production and these activities* was observed in repeatedly frozen and thawed Berkefeld N filtrates of the same strains. The number of strains tested is too small for generalization. Further work is necessary to determine the significance of somewhat stronger activities, particularly of tributyrin hydrolysis, observed in some but not all hemotoxic preparations.

Reducing power for methylene blue.—Reducing power of staphylococci (as shown in table 2) is definitely correlated, in extent and speed of reduction of methylene blue to the leuko-base, with hemotoxin production. All hemo-

* Enzymic activities of Berkefeld filtrates were so slight as to be of questionable significance, and are accordingly not reported in detail here.

TABLE 1.—*Proteolytic, Lipolytic and Carbohydrate—Utilizing Activities of Whole Cultures of Staphylococci*

Strain Number	Cultivation of Enzymic Extract Performed in	Peptone Hydrolysis	Tributyrin Hydrolysis	Acid Formed from Glucose	Hemotoxin
		Amino Nitrogen Per 100 Cc. of Substrate	Amount of N/40 Sodium Hydroxide Required to Neutralize Acid Formed During Hydrolysis in 10 Cc. of Substrate	Amount of N/40 Sodium Hydroxide Required to Neutralize Acid Formed During Glucose Decomposition in 10 Cc. of Substrate	Units Per Cc.
		Mg.	Cc.	Cc.	
40	air	24.37	0.01	0.19	tr ¹ ?*
	20% CO ₂	20.78	—0.01	0.13	0
4	air	15.76	0.01	0.08	tr ¹ ?*
	20% CO ₂	27.24	0.15	0.10	0
7	air	24.01	0.19		2
	20% CO ₂	30.11	0.35	0.18	400
1	air	35.48	0.26	0.12	2
	20% CO ₂	48.02	0.42	0.12	800
Control (medium no enzyme)	air	0.72	0.02	0.01	0

Substrates: amount, 10 cc.
Staphylococcus preparation: amount, 1 cc.
0 = negative result.

* The questionable trace of hemolysis appearing here is probably due to the extremely heavy growth of organisms in soft agar extracts. Tests performed in the ordinary manner with broth cultures exhibit no hemolysis.

toxic strains caused complete reduction of methylene-blue (1–2,000 dilution) in 24 hours. On the other hand, no nonhemotoxic strain produced complete reduction and only an occasional strain exhibited incomplete and much delayed reduction.

TABLE 2.—*Relation of Reducing Activity to Hemotoxin*

No.	Staphylococcus Pigment	Reduction of Methylene-Blue				Hemolysis of Rabbit Corpuscles*
		1 Hour 1–2,000	2 Hours 1–2,000	4 Hours 1–2,000	24 Hours 1–2,000	
1	aureus	0	+	++	++++	+++++
9	aureus	0	++	++	++++	+++++
42	aureus	+	++	++	++++	+++++
43	aureus	0	++	+++	++++	+++++
44	albus	0	++	++	++++	+++++
95	aureus	++	++	++	++++	+++++
87	aureus	0	0	+	++++	++
97	aureus	0	+	+++	++++	++
27	aureus	0	++	+++	++++	+++
49	aureus	0	+++	+++	++++	+++
50	aureus	0	0	++	++++	+++
5	aureus	+	++	+++	++++	+++
6	aureus	0	+++	+++	++++	+++
7	aureus	0	+	+++	++++	+++
37	aureus	+	++	+++	++++	++
38	aureus	0	+++	+++	++++	+++
39	aureus	0	++	+++	++++	+++
33	albus	0	0	++	++++	+++
82	albus	0	+++	+++	++++	++
4	albus	0	0	0	0	0
25	albus	0	0	0	0	0
32	aureus	0	0	0	0	0
46	aureus	0	0	0	0	0
60	albus	0	0	0	0	0
76	aureus	0	0	0	0	0
101	albus	0	0	0	++	0
71	albus	0	0	0	0	0
78	albus	0	0	0	0	0
88	albus	0	0	0	0	0
77	aureus	0	0	0	0	0
48	aureus	0	0	0	0	0
40	albus	0	0	+	0	0
41	albus	0	0	0	+	0
51	citreus	0	0	0	0	0
80	albus	0	6	0	++	0

* 0.5 cc. 48 hour veal infusion broth culture of organism +0.5 cc. diluted methylene blue.
Control: sterile broth, no enzyme—negative.

DISCUSSION

Reducing power of staphylococci for methylene blue is shown, when tested in proper dilution, to be correlated with ability to produce hemotoxin. Lesbré and Jausion⁴ have reported reducing power of staphylococci to be associated with severity of infection from which the organisms are derived and with dermatotoxic power. The virulence of pneumococci for mice has been shown by quantitative determination to be associated with hydrogen peroxide production.⁵ It appears, therefore, that certain so-called "toxic" and invasive properties of microorganisms, namely, hemotoxin production in staphylococcus preparations as well as the virulence of pneumococci, are associated with ability to dehydrogenate in the presence of a hydrogen acceptor. Whether this association of hemotoxic and reducing properties is specific or is simply a coincident expression of metabolic activity of growing organisms is not known.

It is of interest in this connection that hemotoxic properties of staphylococci appear to bear a definite relationship in strength of these substances to certain other toxic properties of the organism. A correlation has been demonstrated between strength of hemotoxic power and dermatotoxic and killing properties (1-2).^{*} The enterotoxic substance of staphylococci, a gastro-intestinal poison for man and monkeys,⁶ has been reported by Woolpert and Dack⁷ to be distinct from the hemotoxin in that all hemolytic strains do not contain demonstrable enterotoxic properties for rhesus monkeys. All enterotoxic strains, conversely, appear to contain hemolysin.

SUMMARY

Measurement of peptone hydrolysis, tributyrin hydrolysis, and glucose utilization as indicated by acid formation, have shown no pronounced or consistent association between extent of these activities and hemotoxin production, (a) in comparison of hemotoxic and nonhemotoxic strains, (b) upon marked enhancement of hemotoxin production with cultivation in an atmosphere containing carbon dioxide.

Hemotoxin production in staphylococci is shown definitely to be correlated with extent and speed of reduction of methylene blue to the leukobase.

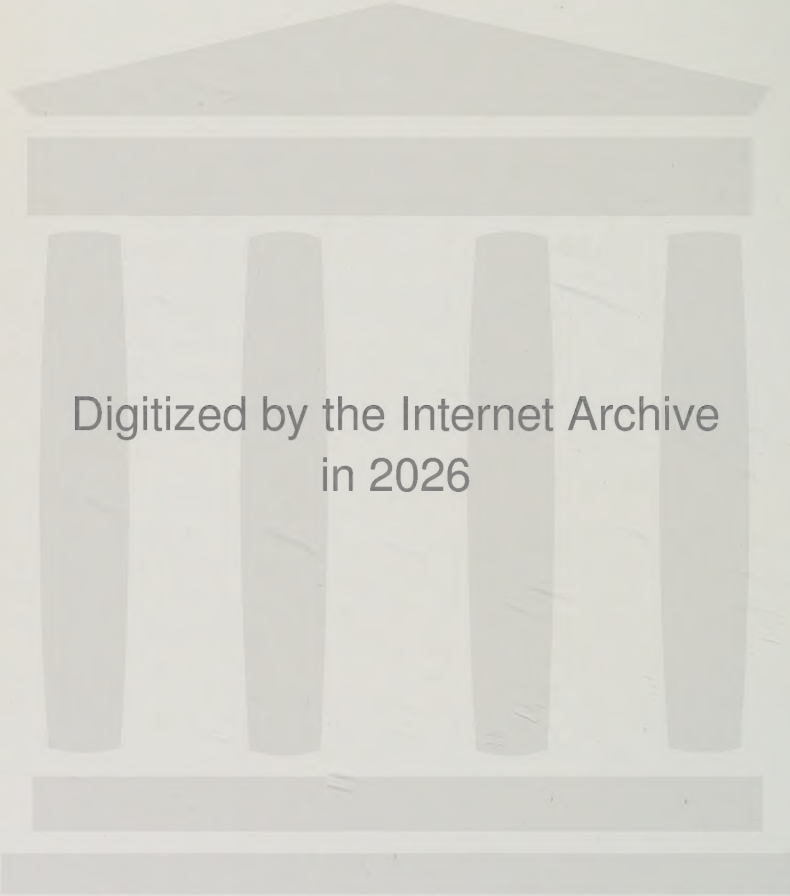
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5. Fujita, A. and Kodama, T.: Biochem. Ztschr. **17**: 277, 1935.

^{*} There is evidence against the unity of these substances (2).

6. Dack, G. M., Cary, W. E., Woolpert, O., and Wiggers, H.: J. Prev. Med. **4**: 167, 1930; Jordan, E. O. and McBroom, J.: Proc. Soc. Exper. Biol. & Med. **29**: 161, 1929.

7. J. Infect. Dis. **52**: 6, 1933.



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